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Evolution in African tropical trees displaying ploidy-habitat association: The genus *Afzelia* (Leguminosae)

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ABSTRACT

Polyploidy has rarely been documented in rain forest trees but it has recently been found in African species of the genus *Afzelia* (Leguminosae), which is composed of four tetraploid rain forest species and two diploid dry forest species. The genus *Afzelia* thus provides an opportunity to examine how and when polyploidy and habitat shift occurred in Africa, and whether they are associated. In this study, we combined three plastid markers (*psbA*, *trnL*, *ndhF*), two nuclear markers (ribosomal *ITS* and the single-copy *PEPC* E7 gene), plastomes (obtained by High Throughput Sequencing) and morphological traits, with an extensive taxonomic and geographic sampling to explore the evolutionary history of *Afzelia*. Both nuclear DNA and morphological vegetative characters separated diploid from tetraploid lineages. Although the two African diploid species were well differentiated genetically and morphologically, the relationships among the tetraploid species were not resolved. In contrast to the nuclear markers, plastid markers revealed that one of the diploid species forms a well-supported clade with the tetraploids, suggesting historical hybridisation, possibly in relation with genome duplication (polyploidization) and habitat shift from dry to rain forests. Molecular dating based on fossil-anchored gene phylogenies indicates that extant *Afzelia* started diverging c. 14.5 or 20 Ma while extant tetraploid species started diverging c. 7.0 or 9.4 Ma according to plastid and nuclear DNA, respectively. Additional studies of tropical polyploid plants are needed to assess whether the ploidy-habitat association observed in African *Afzelia* would reflect a role of polyploidization in niche divergence in the tropics.

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1. Introduction

The evolutionary mechanisms that have driven the diversification of rain forest species are still poorly understood. Evidence from recent biogeographical and phylogenetic insights indicate that Guineo-Congolian rain forests (i.e. lowland rain forests from West and Central Africa; White, 1983) are contain both recent and relict species and it is difficult to evaluate the influence of past environmental changes on the evolutionary history of this melting-pot of lineages and taxa (Plana, 2004). In addition, factors as diverse as reproductive isolation, ecological gradients and/or polyploidization may have played a key role on current patterns of biodiversity (Baraloto et al., 2012; Dauby et al., 2014).

It is now widely recognized that polyploidy is an important evolutionary mechanism in plant diversification (Soltis et al., 2014). It can confer new adaptive capacities and be particularly important in a changing environmental context (Ramsey and Schemske,

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2002; Soltis et al., 2004, 2007). Polyploid species are often characterized by a broader ecological tolerance than their diploid progenitors (Otto and Whitton, 2000). Nevertheless, these predictions seem to be species-specific and need to be better examined in an evolutionary framework (e.g. Baack and Stanton, 2005; Sampoux and Huyghe, 2009; Ramsey, 2011; McIntyre, 2012; Theodoridis et al., 2013). In the arctic flora, allopolyploid plants dominate in deglaciated regions, suggesting that polyploidization could favour adaptation to new or harsh habitats (Brochmann et al., 2004). Whether this could also hold in tropical biomes is unknown, but in Africa, palynological data showt cyclic vegetation changes from rain forest to savannah and vice versa during the Quaternary (Dupont et al., 2000), thus offering conditions that may have promoted polyploids if they are better adapted to changing conditions.

To our knowledge, polyploidization has been rarely documented in tropical trees except in some species belonging to the genera *Shorea* (Dipterocarpaceae; Jong and Lethbridge, 1967), *Terminalia* (Combretaceae; Ohri, 1996), *Polylepis* (Rosaceae; Schmidt-Lebuhn et al., 2010) and *Adansonia* (Bombacaceae; Pettigrew et al., 2012). In the Legume family, where ancient polyploidization events might have played an important role for its early diversification (Pfeil et al., 2005; Cannon et al., 2010, 2015; Doyle, 2012), polyploid tropical trees have been found in the genera *Acacia* (Assoumane et al., 2013), *Leucaena* (Hughes et al., 2002), and recently in *Afzelia* using flow cytometry and nuclear microsatellites (Donkpegan et al., 2015).

The Palaeotropical genus *Afzelia* (tribe Detarieae) has been revised repeatedly by taxonomists (Chevalier, 1940; Léonard, 1950; INEAC, 1952; Keay, 1954; Aubréville, 1959, 1968, 1970; Satabie, 1994). At present, most taxonomists agree that the genus contains 11 species, even if it remains difficult to unambiguously delimit them in the field using morphological traits (Donkpegan et al., 2014). Four species grow in Southeast Asia: *A. xylocarpa* (Kurz) Craib, *A. rhomboidea* (Blanco) S.Vidal, *A. javanica* (Miq) J. Léonard and *A. palembanica* Baker, and seven are distributed in sub-Saharan Africa: *A. bipindensis* Harms, *A. bella* Harms, *A. pachyloba* Harms, *A. parviflora* (Vahl) Hepper, *A. africana* Sm. ex Pers., *A. quanzensis* Welw., and *A. peturei* De Wild. Interestingly, in Africa, ecological niche and ploidy levels appear linked, since diploidy is found in dry forest species allopoatrically distributed (*A. africana* and *A. quanzensis*), whereas tetraploidy is associated with rain forest sympatrically distributed species (*A. bipindensis, A. bella, A. pachyloba,* and *A. parviflora*) (Donkpegan et al., 2015). Whether morphological differentiation between species is also linked to ploidy level and habitats has not been documented. However, the absence of a reliable phylogenetic framework limits our ability to further infer the evolutionary relationships between these species.

The present study aims to better understand the drivers of plant diversification in tropical Africa, with a focus on the role of polyploidy, using the genus *Afzelia* as a model. More specifically, we address the following questions: (1) Do polyploid species form a single clade, suggesting a potentially unique polyploidization event, or can we document repeated polyploidization events, possibly driving particular niche adaptation? (2) When did *Afzelia* diversification occur, in particular in relation to ploidy and habitat shifts? (3) Is morphological differentiation related to phylogenetic divergence and/or difference of ploidy and habitat? To answer these questions we combined morphological and genetic data, used both plastid (*psb*A, *trn*L, *ndh*F) and nuclear (ribosomal *ITS* and *PEPC*-E7) DNA sequences to infer phylogenetic relationships, and relied on High Throughput Sequencing (HTS) of the plastome of a subset of samples to increase phylogenetic resolution and improve molecular dating.

2. Materials and methods

2.1. Sampling and DNA extraction

For genetic analyses, leaflets and cambium samples (dehydrated with silicagel) were collected on 43 specimens representing six African *Afzelia* species (39 specimens from 11 countries), two Asian *Afzelia* species (three specimens), and *Intsia bijuga* (one specimen from Madagascar) which is sister to the genus *Afzelia* (Mackinder et al., 2013) (Table 1). Species identification in the field was performed using the criteria described in Donkpegan et al. (2014). Only one sample per taxon was included for whole plastome sequencing (six African and two Asian *Afzelia* species, and *Intsia bijuga*; Table 1), while 15–31 samples were sequenced at particular plastid and/or nuclear regions (Table 1). DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. For plastome High Throughput Sequencing (HTS), the CTAB protocol was used (Doyle and Doyle, 1987) coupled with QIAquick purification kits (Qiagen), and followed by Qubit® 2.0 Fluorometer quantification (Life Technologies, Invitrogen, Foster City, USA).

2.2. DNA polymorphism in plastid and nuclear regions assessed by Sanger sequencing

For 31 samples, two plastid DNA (pDNA) intergenic regions (*psbA-trnH* and *trnL*) and two plastid coding genes (*ndHF84F-ndHF713R* and *matK1R*-3F) were amplified and sequenced using universal primers (Kim and Jansen, 1995; Chase et al., 2007; Bruneau et al., 2008). Then, *matK* sequences were discarded due to their lower sequencing success and polymorphism in comparison to other loci. The PCR reaction used 1.5 μL of DNA template (20–50 ng), 0.1 μL of *Taq* polymerase (Qiagen), 2.5 μL of PCR buffer, 1 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTPs (10 mM), 0.25 μL of each primer (10 mM), and 19.375 μL of H_2O. The PCR cycling profile included 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. Sanger sequencing was performed on a 3100 Genetic Analyzer (Applied Biosystems) in the ULB-EBE platform (Brussels, Belgium).

For the nuclear genome (nDNA), amplification and polymorphism were tested for 13 single-copy loci: *ADH, GScp, ADHc, Rnl1, TPI 6rn/4rn, LF4 Cl1R, LF4 Cl4R, Agt1, Apg1, At103 PEPC* E1/E2, *PEPC* E2/E3 and *PEPC* E7 (Li et al., 2008; Duminil et al., 2015), and two nuclear ribosomal internal transcribed spacers: ITS1 and ITS2 (Li et al., 2008). Based on amplification success and polymorphism, only *PEPC* E7 and *ITS2* were retained and successfully sequenced for 31 and 15 samples, respectively. PCR reactions followed the pDNA protocol, but using 0.125 μL of *Taq* polymerase Phusion (Qiagen), with 5 μL of PCR buffer.

Sequences were manually edited and aligned using the MUSCLE algorithm (Edgar, 2004) implemented in CodonCode Aligner 3.7.1 (CodonCode, St. Louis, Missouri). For nuclear *PEPC* E7 sequences, where heterozygous sites were sometimes observed, we used PHASE v2.1.1 algorithm (Stephens et al., 2001) implemented in DNASP v.5.10 program (Librado and Rozas, 2009) to infer the two alleles present in heterozygous individuals at each locus through Bayesian inference (Garrick et al., 2010). Indels were coded in FastGap 1.0.8 (Borchsenius, 2007).

2.3. Phylogenetic reconstructions based on plastid and nuclear regions

Phylogenetic relationships were inferred using Maximum Parsimony (MP) and Bayesian Inference (BI) methods, considering separately (i) the three concatenated pDNA loci, (ii) *ITS2*, and (iii) *PEPC*

Table 1
Material used for the molecular analyses of *Afzelia*.

Species	Country	Location	Vouchers	Collectors	Herbarium	Latitude	Longitude	Markers successfully sequenced
A. quanzensis 2X	Kenya	Gede	AD522	Donkpegan S.L.A.	BRLU	−3.30295	39.9845	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. quanzensis	Kenya	kwalé	AD523	Donkpegan S.L.A.	BRLU	−4.18279	39.4405	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. quanzensis	Kenya	Gede	AD518	Donkpegan S.L.A.	BRLU	−3.27098	39.98244	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. quanzensis	Kenya	Gede	AD519	Donkpegan S.L.A.	BRLU	−3.30439	39.98602	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. quanzensis	Kenya	Gede	AD520	Donkpegan S.L.A.	BRLU	−3.30715	39.98181	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. quanzensis	Kenya	Gede	AD521	Donkpegan S.L.A.	BRLU	−3.30324	39.98324	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. quanzensis	Kenya	Gede	AD524	Donkpegan S.L.A.	BRLU	−3.3036	39.99108	*ITS2*
A. quanzensis	Kenya	Witu	AD889	Donkpegan S.L.A.	BRLU	−2.37063	40.48026	plastome
A. africana 2X	Benin	Lama	AD17	Donkpegan S.L.A.	BRLU	6.97694	2.13496	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. africana	Benin	Pénéssoulou	AD88	Donkpegan S.L.A.	BRLU	9.26313	1.50939	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. africana	Benin	Lama	AD20	Donkpegan S.L.A.	BRLU	6.97681	2.13497	*ITS2*
A. africana	Benin	Lama	AD21	Donkpegan S.L.A.	BRLU	6.97673	2.13515	*ITS2*
A. africana	Benin	Lama	AD26	Donkpegan S.L.A.	BRLU	6.97659	2.13529	*ITS2*
A. africana	Benin	Lama	AD27	Donkpegan S.L.A.	BRLU	6.97603	2.14591	Plastome
A. africana	Senegal	Casamance	AD123	Donkpegan S.L.A.	BRLU	12.49356	−16.21985	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. africana	Senegal	Casamance	AD125	Donkpegan S.L.A.	BRLU	12.88330	−16.13330	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. africana	Burkina-Faso	Pama	ADJu1	Donkpegan S.L.A.	BRLU	11.25120	0.78157	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. africana	Burkina-Faso	Pama	ADJu2	Donkpegan S.L.A.	BRLU	11.32541	0.82232	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. africana	Ghana	Akwapim	NB-0522	Bourland N.	BRLU	5.95632	−0.16978	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bipindensis 4X	Cameroon	Makalaya	AD270	Donkpegan S.L.A.	BRLU	3.28469	14.33968	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bipindensis	Republic of Congo	Loundoungou	JFG-744	Gillet J-F	BRLU	2.3776	17.0641	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bipindensis	Republic of Congo	Pokola	JFG-864	Gillet J-F	BRLU	1.32573	16.39493	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bipindensis	Cameroon	Makalaya	AD455	Donkpegan S.L.A.	BRLU	3.28157	14.33568	*ITS2*
A. bipindensis	Gabon	Bambidie	AD626	Donkpegan S.L.A.	BRLU	−0.73304	12.91774	Plastome
A. pachyloba 4X	Cameroon	Maan	AD411	Donkpegan S.L.A.	BRLU	2.24512	10.47447	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. pachyloba	Cameroon	Maan	AD413	Donkpegan S.L.A.	BRLU	2.25146	10.46291	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. pachyloba	Cameroon	Campo	AD485	Donkpegan S.L.A.	BRLU	2.46473	9.90157	Plastome, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. pachyloba	Cameroon	Campo	AD486	Donkpegan S.L.A.	BRLU	2.46573	9.90142	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bella 4X	Cameroon	Mamfé	AD440	Donkpegan S.L.A.	BRLU	5.738582	9.555438	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bella	Cameroon	Mamfé	AD442	Donkpegan S.L.A.	BRLU	5.750495	9.547516	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bella	Cameroon	Korup	PM4985	Parmentier I.	BRLU	5.062	8.854	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bella	Cameroon	Mamfé	AD436	Donkpegan S.L.A.	BRLU	5.743841	9.566711	plastome
A. bella	Ghana	Atiwa	NB0489	Bourland N.	BRLU	6.22959	−0.54069	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bella var gracilior	Liberia	Gbarpolu	HBAD27/10254	Jongkind C.C.H.	BRLU/BR	7.45	−10.33	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. bella var gracilior	Guinea	Nzérékoré	HBAD28/27	Haba O-O.	BRLU/BR	7.70	−8.32	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. parviflora 4X	Sierra-Leone	Tonkolili	HBAD29/1438	Van der Burgt X. M.	BRLU/BR	8.94861	−11.76861	Plastome, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. parviflora	Liberia	Gbarnga	HBAD30/10979	Jongkind C.C.H.	BRLU/BR	7.183	−9.300	*ITS2*, *psbA*, *ndhF*, *trnL* and *PEPC* E7
A. rhomboidea 2X	Indonesia	East Kalimatra	HBAD160/ AA1567	Ambri et al.	BRLU/ WAG	0.49	117.33	Plastome
A. xylocarpa 2X	Cambodia	Siem Riep	Axy1	Mouget J.	BRLU	13.45305	103.89966	*psbA*, *ndhF*, *trnL* and *PEPC* E7
A. xylocarpa	Cambodia	Siem Riep	Axy2	Mouget J.	BRLU	13.45398	103.89960	Plastome, *psbA*, *ndhF*, *trnL* and *PEPC* E7
Intsia bijuga 2X	Madagascar	Tamatave	HBAD32/1469	Schatz G.E & D'arcy W.	BRLU/BR	−18.9	49.13	*ITS2*, plastome, *psbA*, *ndhF*, *trnL* and *PEPC* E7

E7. Applying MP principles, median-joining networks, based on single-nucleotide polymorphisms (SNPs) and insertions–deletions (indels), were represented using the software Network 4.6.1.3 (Bandelt et al., 1999). For the BI approach, we used MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), implementing the following evolutionary models selected according to the Bayesian Information Criterion (BIC, Schwartz, 1978) provided by jModelTest 2.1.7 (Darriba et al., 2012): F81 for pDNA, HKY + G for *ITS2*, K80 for *PEPC* E7 2nd codon position, and JC for *PEPC* E7 1st and 3rd codon positions. The Binary model implemented in MrBayes for restriction sites was used for the indel binary dataset with the command "coding = variable" to adjust for characters not included in this matrix, as suggested by Ronquist and Huelsenbeck (2003). The Metropolis Coupled Markov Chains (MCMC) with Monte Carlo simulations were run for 10,000,000 generations, with a tree sampled every 1000 generations. Chain stationarity was checked using the average standard deviation of split frequencies (Gelman and Rubin, 1992), and was achieved after 5000 generations (burn-in). A 50% majority rule consensus tree was constructed from the remaining trees.

2.4. Plastome sequencing using HTS tools and bioinformatics

High Throughput Sequencing technology was applied to obtain more polymorphic pDNA data for each of nine species in order to improve phylogenetic resolution and perform molecular dating. We used a cost-effective enrichment through hybridisation capture of plastome at deep multiplexing levels, following Mariac et al. (2014). In summary, DNA extracts were sheared using a Bioruptor® Pico (Diagenode SA., Liège, Belgium) to yield sonicated fragments around ca. 400 bp. Sheared and sized DNA was then repaired and tagged using 6-bp barcodes for multiplexing all the samples (Rohland and Reich, 2012), after AMPure XP bead-based sample clean-up steps (Agencourt Bioscience, Beckman Coulter, Brea, USA). To capture chloroplast sequences directly by hybridisation from the genomic DNA libraries, we used biotinylated probes pre-

viously produced in the laboratory for another Leguminosae tree from the same Detarieae clade, *Guibourtia tessmannii*, using 18 long-range universal PCR primers combinations (Uribe-Convers et al., 2014) with the LongAmp® Taq PCR Kit (New England Biolabs, Beverly, USA). Real-time PCRs were then performed to generate the plastome-enriched genomic libraries, on a StepOnePlus (Applied Biosystems, Foster City, USA). Paired-end sequencing (2 × 150 bp) was performed on an Illumina MiSeq with reagent kit V2 at the GIGA platform (Liège, Belgium), using *c.* 1/100th of the run for each plastome enriched sample.

The plastome enrichment phase does not guarantee that all parts of the plastome are captured, given that we used probes made from another related species. Thus the protocol of genomic libraries preparation was also applied without enrichment to reconstruct a reference plastome on one sample of *A. africana* from Benin, using *c.* 1/30th of the MiSeq run. An independent genomic library was also obtained for *Intsia bijuga*.

Demultiplexing based on the 6-bp barcodes was performed using the freely available python script Demultadapt (https://github.com/Maillol/demultadapt), using a 0-mismatch threshold. Adapters and low-quality bases were removed using Cutadapt 1.2.1 (Martin, 2011) with the following options: quality cut-off = 20, minimum overlap = 7 and minimum length = 35. Reads with a mean quality lower than 30 were discarded afterwards using a freely available perl script (https://github.com/SouthGreenPlatform/arcad-hts/blob/master/scripts/arcad_hts_2_Filter_Fastq_On_Mean_Quality.pl). The software Mitobim 1.5 (Hahn et al., 2013) was used to reconstruct the plastid genome of *A. bipindensis*, assembling the reads from the non-enriched genomic DNA library. Mitobim mapped these reads using MIRA 3.4.1.1 (Chevreux et al., 1999) to highly conserved regions of the closely related reference genome available in GenBank: *Tamarindus indica* (Leguminosae Detarieae; NC_026685.1). The mapping of plastid DNA enriched libraries was performed using Bwa Mem 0.7.5a-r405 (Li and Durbin, 2009) with -M and -B 4 options, using the plastid reference genomes reconstructed in the previous steps with the genomic library of *A. bipindensis*. Samtools 1.1 (Li and Durbin, 2009) was used to generate a mpileup file with option –B, following Scarcelli et al. (2015). We used Varscan 2.3.7 (Koboldt et al., 2012) to call SNPs (option –min-var-freq set to 50%) before converting it to a fasta multi-alignment file.

Plastome sequences from *Afzelia* and *Intsia* were aligned in MEGA 5.0.3 (Tamura et al., 2011). The model JVM + G was chosen using jModelTest 2.1.7 (Darriba et al., 2012). The appropriate code for this model was imported from the BEAST webpage (http://beast.bio.ed.ac.uk/substitution-model-code) and we modified the input file accordingly.

2.5. Fossil calibration and molecular dating

Dated phylogenies were reconstructed for (i) the nine plastomes and (ii) the 17 haplotypes of the *PEPC* E7 with the Bayesian MCMC analysis implemented in BEAST v1.7.4 (Bayesian Evolutionary Analysis by Sampling Trees; Drummond and Rambaut, 2007). To time-calibrate the phylogenies we considered an *Afzelia* fossil dated to 27.23 Ma (Pan et al., 2010) which provides a minimal age for the divergence between *Intsia* and *Afzelia*. As these sister genera belong to the large Amherstieae clade (Bruneau et al., 2008), they must have diverged more recently than the age of Amherstieae, estimated at 52.1 Ma by Bruneau et al. (2008). Thus, we anchored the phylogenies by setting the age of the divergence between *Intsia* and *Afzelia* as a gamma distributed random variable offset by 27.23 Ma (shape = 4, scale = 2), giving a 95% confidence interval between 29.5 and 42.8 Ma. An XML file was generated using BEAUti (Bayesian Evolutionary Analysis Utility). An uncorrelated lognormal relaxed clock model and a Yule process of speciation were applied. Two MCMC analyses were run for 50 million generations each, sampling trees at 1000 step intervals. We used Tracer 1.4 (Drummond and Rambaut, 2007) to assess convergence, estimate Effective Sample Sizes (ESS), and examine the posteriors of all the parameters. Runs were associated with LogCombiner after removing the first 10,000 samples as burn-in. Mean heights were taken in TreeAnnotator 1.4.8 (Drummond and Rambaut, 2007), and trees were plotted in FigTree 1.1.2 (http://tree.bio.ed.ac.uk/software/figtree/).

2.6. Morphometric analyses

Morphological analyses were performed on 38 herbarium specimens corresponding to six African *Afzelia* taxa (*A. africana*, *A. bella var. bella, A. bella var. gracilior, A. bipindensis, A. parviflora,* and *A. quanzensis* – Table 2), for which fully opened flowers could be studied (no fertile vouchers were available for *A. peturei* and *A. pachyloba* specimens). Fifteen vegetative characters related to leaves (8 quantitative, 7 qualitative) and seven floral traits related to petal, pedicel and bracteole (4 quantitative, 1 qualitative) were measured (see Fig. 1 and Table 3). One open flower per specimen was rehydrated in boiling water for 30 s to 1 min to facilitate dissection in distilled water. Quantitative characters were measured with graph paper, recorded as numeric (continuous or discrete) values and standardized (Poulsen and Nordal, 2005; Marcysiak et al., 2007), whereas qualitative characters were recorded as factors and not standardized (Sneath and Sokal, 1973).

To investigate independently the assignation of specimens to species and to identify discriminant variables, a Hill-Smith analysis was first performed (Hill and Smith, 1976), using the function *dudi.hillsmith* in the Ade4 package (Chessel et al., 2004; Dray et al., 2007) available in R 3.1.2 (R-Core Team, 2013). Data were ordinated on a biplot graph using a principal coordinate analysis (PCoA) and the Gower index. These analyses were first performed separately on the vegetative and floral traits, before combining them.

Quantitative variables were examined using the Shapiro-Wilk test of normality (Royston, 1982) available in the package Stats in R 3.1.2. For 10 out of the 13 quantitative characters, normality was confirmed and a one-way analysis of variance (ANOVA) was performed (Sokal and Rohlf, 1995). For the three non-normal variables, the non-parametric Kruskal-Wallis test was performed (Hollander and Wolfe, 1973). For each test, the null hypothesis (H_0) was "no difference between means or medians for each group of morphospecies". When the null hypothesis was rejected, we conducted multiple comparisons between means and medians to determine which groups were different using the Tukey's Honestly Significant Difference test (Tukey, 1977) and the test of *kruskalmc* (Siegel and Castellan, 1988) available in the PGIRMESS package (Giraudoux, 2013).

3. Results

3.1. DNA polymorphism

The combined pDNA (*psbA*, *ndhF* and *trnL*) matrix comprised 1286 bp and 31 samples. It included 21 coded indels and 22 single nucleotide polymorphic sites of which 90% were parsimony-informative sites (Table 4). A total of 12 pDNA haplotypes were identified for *Afzelia*, using the MP approach developed in the software NETWORK. Haplotypes of *A. quanzensis* (H1 and H2) and *A. africana* (H4, H5 and H12) are distinct and never shared with other rain forest species; while some haplotypes (H6 and H7) are shared between rain forest species (Fig. 2A and B). Private haplotypes

Table 2
Material used for the morphometric analyses of African species of *Afzelia*.

Taxon	Geographic origin	Herbarium	Voucher	Collectors
A. africana 1	Cameroon	WAG	15,535	Meijer W.
A. africana 2	Cameroon	WAG	4708	Geerling C.
A. africana 3	Cameroon	WAG	13,335	na
A. africana 4	Benin	BR	8	Pierret H.
A. africana 6	Senegal	BR	7265	Berhaut
A. africana 8	DRC	BR	2218	Lebrun J.
A. bella var bella 1	Cameroon	BR	8307	Bos J.J.
A. bella var bella 2	Gabon	BR	14,446	Breteler F.J.
A. bella var bella 3	Cameroon	BR	942	van der Burgt X.M.
A. bella var bella 4	DRC	BR	9785	Louis J.
A. bella var bella 5	DRC	BR	1027	Lebrun J.
A. bella var gracilior 1	Ghana	BR	5811	Andok J.E.
A. bella var gracilior 2	Guinea-Conakry	BR	27	Haba
A. bella var gracilior 3	Liberia	WAG	10,254	Jongkind C.C.H.
A. bella var gracilior 4	Guinea-Conakry	WAG	27	Haba O.O. (WAG. 1704893)
A. bipindensis 1	Cameroon	BR	8033	de Wilde J.J.F.E.
A. bipindensis 3	Gabon	BR	7806	Le Testu G.
A. bipindensis 4	Gabon	BR	na	na
A. bipindensis 5	RCA	BR	3930	Le Testu G.
A. bipindensis 6	RCA	BR	4664	Le Testu G.
A. bipindensis 7	Gabon	BR	344	Arends J.C./Louis A.M./de Wilde J.J.F.E.
A. bipindensis 8	Gabon	BR	7185	Le Testu G.
A. bipindensis 9	DRC	BR	1341	na
A. bipindensis 10	DRC	BR	35	Hardy Y./Mwene Ditu
A. quanzensis 1	Mozambique	BR	7529	Jansen P.C.M.
A. quanzensis 2	Zambia	BR	898,624	Milne-Redhead E.
A. quanzensis 3	Zambia	BR	1233	Milne-Redhead E.
A. quanzensis 4	Angola	BR	1138	Raimundo F./Matos e Figueira C.
A. quanzensis 5	Kenya	BR	542	Reitsma J.M.
A. parviflora 1	Liberia	BR	10,979	Jongkind C.C.H.
A. parviflora 2	Siera-Leone	BR	1438	van der Burgt X.M.
A. parviflora 3	Liberia	BR	3751	de Wilde J.J.F.E./Voorhoeve A.G.
A. parviflora 4	Liberia	BR	159	Konneh P.V.
A. parviflora 5	Liberia	BR	288	Voorhoeve A.G.
A. parviflora 6	Liberia	WAG	2935	Bos J.J.
A. parviflora 7	Liberia	WAG	1071	Voorhoeve A.G.
A. parviflora 8	Liberia	WAG	922	Bos J.J.
A. parviflora 9	Liberia	WAG	1226	Voorhoeve A.G.

appear however for *A. bella* (H8 and H9) and *A. parviflora* (H10) (Fig. 2B).

The nDNA *PEPC* E7 alignment of 31 samples reached 558 bp in length, with 31 polymorphic sites. Heterozygous sites (10 sequences with one heterozygous site and one sequence with two heterozygous sites) were treated with the PHASE algorithm which provided a total of 17 haplotypes. Despite the fact that the *PEPC* E7 haplotype H5 is shared between all *Afzelia* rain forest species, each of these species harbours private haplotypes: H4 and H6 for *A. parviflora,* H7 for *A. bipindensis*, H8 and H9 for *A. pachyloba,* H10, H11 and H12 for *A. bella* (Fig. 2A and C). Haplotypes of *A. africana* (H13 and H14) and *A. quanzensis* (H15, H16 and H17) are distinct and never shared with other rain forest species, as previously noted for the pDNA data (Fig. 2C).

Regarding the nDNA *ITS2* region aligned on 471 bp, 11 haplotypes were defined among the 15 individuals successfully sequenced. Despite the lower sampling in comparison to other markers, this locus revealed a high level of polymorphism with 125 polymorphic sites among which 73 were parsimony-informative (Table 4). There was no shared haplotype between species but the limited sample size may have prevented us detecting shared haplotypes.

The genomic library of *A. africana* used for reconstructing the reference plastome (size of 159,728 bp) contained 1,250,176 R1-R2 paired reads, of which 2.77% appeared to be of plastid origin, making an average depth of 30X. For the nine enriched libraries, on average, 336,590 R1-R2 paired reads (SD: 230,665) were obtained for each plastome capture, of which 97.17% (stdev 1.5%) appeared to be of plastid origin, illustrating a good yield for pDNA enrichment. The average depth was 304X (mean SD: 195) and the mean coverage at 10× was around 78.8% (SD: 0.05%). After SNP (Single Nucleotide Polymorphic) calling, 579 SNPs were retained and used for reconstructing fasta files of complete plastomes, which formed nine haplotypes.

3.2. Phylogenetic relationships and timing of diversification of the genus Afzelia

Bayesian phylogenetic reconstructions indicate that while each African diploid species (*A. africana* and *A. quanzensis*) appears monophyletic, the tetraploid rain forest species are paraphyletic and embedded within a clade excluding *A. africana* and the Asian species (Appendix 1). An important incongruence between pDNA and nDNA datasets appears for the position of the *Afzelia quanzensis* clade: the latter belongs to a clade including all tetraploid rain forest species according to pDNA (including the whole plastome phylogeny), while it is not related to tetraploids but appears sister to *A. africana* according to the *PEPC* E7 and *ITS2* phylogenies (Appendix 1). The two Asian *Afzelia* species form a clade according to the whole plastome phylogeny but their divergence from African *Afzelia* is not well resolved: they could be sister to the clade of diploid African species or to the clade of tetraploid species according to the *PEPC* E7 phylogeny. According to the whole plastome phylogeny they appear sister to the clade including tetraploids and *A. quanzensis*, where *A. africana* is the most basal (Fig. 3A).

The diversification of *Afzelia* seems to have started in the early or mid-Miocene, the posterior mean age of the common ancestor

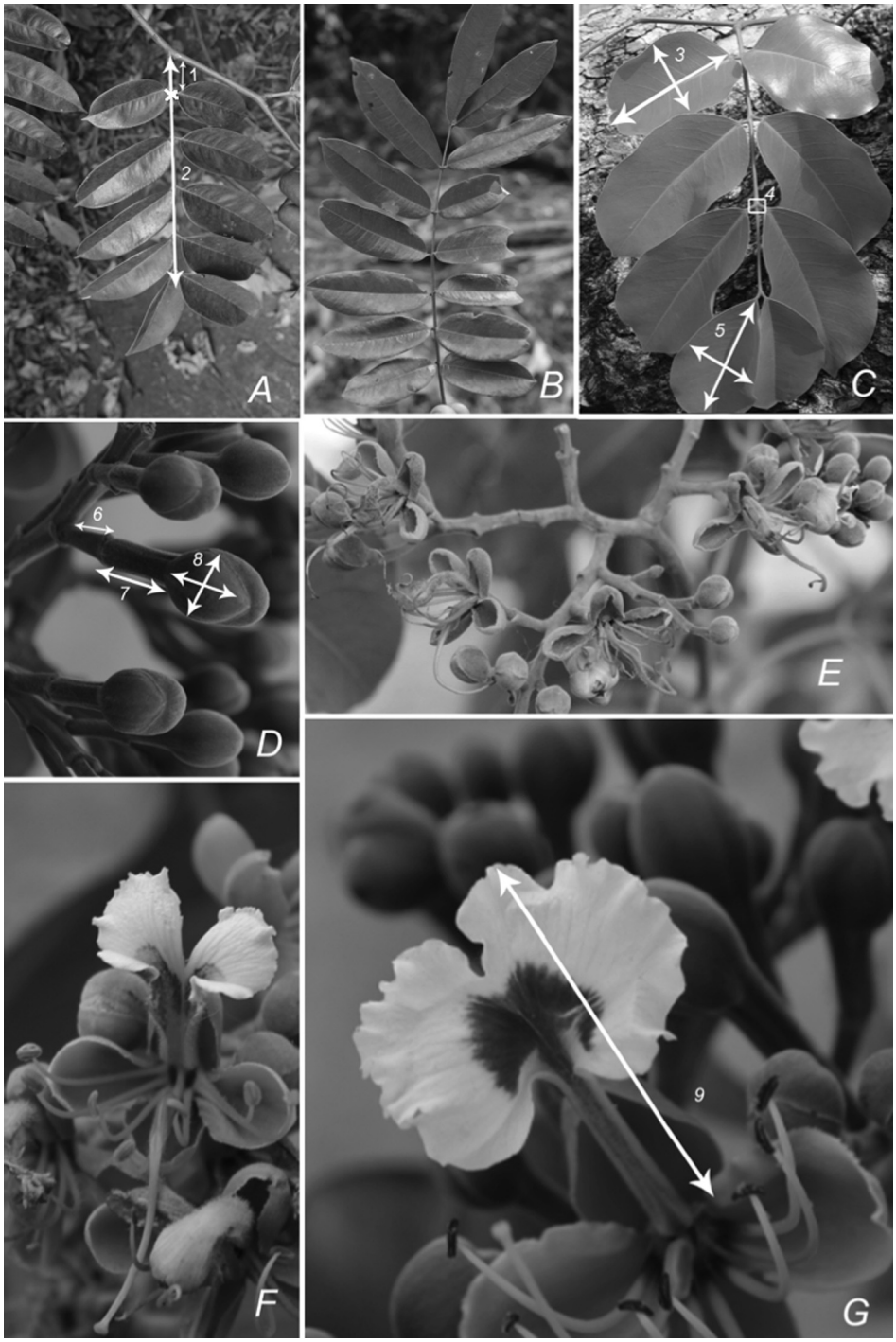


Fig. 1. Illustration of some of the main vegetative and floral characters used to identify African *Afzelia* species: *A. bipindensis* (A), *A. pachyloba* (B), *A. africana* (C, E, F), and *A. bella* (D, G). 1: L_qt_Pl, Petiole length; 2: L_qt_Sl, Spine lenght; 3: L_qt_RlLl, Ratio lenght/width inferior leaflet; 4: L_qt_Pel, Petiolule length; 5: L_qt_RSLl, Ratio lenght/width superior leaflet; 6: F_qt_Pedl, Pedicel length; 7: F_qt_Rl, Receptacle length; 8: F_qt_RlBr, Ratio length/width sepals; 9: F_qt_Pl, Petal length.

(MRCA) of *Afzelia* being estimated at 14.5 Ma (95% highest posterior density HPD between 6.7 and 27.2 Ma) according to plastomes, and 20 Ma (95% HPD between 11.0 and 32.0 Ma) according to *PEPC* E7 (Fig. 3A and B). The Asian species sequenced would have diverged just afterwards (12.5 Ma or 16.8 Ma ago according to plastomes and *PEPC* E7 data, respectively). The diversification of tetraploid rain forest *Afzelia* would have started in the late Miocene (age of MRCA of haplotypes estimated at 7.0 Ma and 9.4 Ma according to plastomes and *PEPC* E7, respectively). Although pDNA and nDNA data diverge regarding the position of *A. quanzensis*, they both situate the divergence between *A. quanzensis* and *A. africana* in the mid or late Miocene (14.5 Ma and 8 Ma according to plastomes and *PEPC* E7, respectively) (Fig. 3A and B).

3.3. Morphologic differentiation and species delimitation

The projection of the PCoA using the two first axes (axis 1:11.6% and axis 2:8.86%) for the floral variables measured on five *Afzelia*

Table 3
Morphometric variables assessed for the study of African species of *Afzelia*.

N°	Characters	Codes	States
1	Leaf length	L_qt_Ll	Continuous (mm)
2	Spine length	L_qt_Sl	Continuous (mm)
3	Petiole length	L_qt_Pl	Continuous (mm)
4	Number of leaflets	L_qt_Len	Discrete (number)
5	Lower leaflets aspect	L_ql_Lela	Shiny; dull
6	Higher leaflets aspect	L_ql_Lehi	Shiny; dull
7	Petiolule length	L_qt_Pel	Continuous (mm)
8	Number secondary veins	L_qt_NSV	Discrete (number)
9	Vein aspect	L_ql_VA	Coriaceous; fine
10	Ratio length/width superior leaflet	L_qt_RSLl	Continuous
11	Ratio length/width inferior leaflet	L_qt_RILl	Continuous
12	Leaflets general form	L_ql_Legf	Elliptic; oval; oblong; falciform; obovate
13	Leaflets apex form	L_ql_Leaf	Scalloped; mucronate apex; acuminate; mucronate apex with black spot
14	Leaflets basal form	L_ql_Lebf	Rounded; attenuated; truncated
15	Translucent dots	L_ql_Letd	Yes; no
16	Petal length	F_qt_Pl	Continuous (mm)
17	Petal hairs	F_ql_Ph	Pubescent; glabrous; downy
18	Pedicel length	F_qt_Pedl	Continuous (mm)
19	Receptacle length	F_qt_Rl	Continuous (mm)
20	Ratio length/width sepals	F_qt_RLlBr	Continuous
21	Ovary hairs	F_ql_Oh	Everywhere pubescent; glabrous; pubescent on 1 or 2 edges only; pubescent on one side only; downy; pubescent at the base
22	Ovary length	F_qt_Ol	Continuous (mm)

Continuous variables were measured in mm; L = Leave; F = Flower; qt = quantitative and ql = qualitative.

species (*A. africana*, *A. bella*, *A. bipindensis*, *A. parviflora*, *A. quanzensis*) showed that *A. africana* and *A. bipindensis* are well differentiated, whereas *A. bella*, *A. parviflora* and *A. quanzensis* present overlapping morphological traits (Fig. 4A). Similar results were obtained with the other axes.

By contrast the vegetative characters provided distinct groups delimiting each species, with the exception of *A. bipindensis* that overlaps with the two varieties of *A. bella* included in the analysis (PCoA axis 1: 21.23% and axis 2: 10%; Fig. 4B). The main discriminant variable is the general form of leaflets: elliptic or oval for *A. africana* and *A. quanzensis*, oblong-falciform for *A. parviflora*, oblong and oblong-obovate for *A. bella* and *A. bipindensis*. In addition, dry forest species (*A. africana* and *A. quanzensis*) are clearly segregated from rain forests species (*A. bella*, *A. bipindensis*, and *A. parviflora*) along the first PCoA axis. This same pattern is also observed by combining vegetative and floral variables for all these five species. The main discriminant variables which separate dry forest versus rain forest species are, respectively: (i) the rounded versus truncated-attenuated basal leaflet shape; (ii) at the distal end of the leaflets, the scalloped versus acuminate and mucronate (sometimes with black spots) (Fig. 4B).

The pairwise comparisons of means or medians using the Tukey-Kramer or Kruskal-Wallis methods revealed significant differences among species for all quantitative variables except the length of pedicel and the ratio length/width of bracteoles (Table 5). By contrast, few characters differed significantly between dry and rain forest species.

4. Discussion

4.1. Phylogenetic relationships and polyploid evolution in the genus *Afzelia*

Our study reveals a discrepancy between nuclear and plastid phylogenies reconstructed within the genus *Afzelia* (Fig. 2). According to the two nDNA markers, there is a clear separation between two African clades: one with four tetraploid species (*A. bella*, *A. bipindensis*, *A. pachyloba* and *A. parviflora*), an the other with diploid ones *A. africana* and *A. quanzensis* (Fig. 3A). On the other hand, the three plastid regions reveal that the diploid species *A. quanzensis*, although monophyletic, is included in a clade grouping the tetraploid species, rather than sister to *A. africana*; a topology confirmed by whole plastome sequencing (Fig. 3A). While reciprocal monophyly is observed for the two diploid species, all four tetraploid species appear paraphyletic and share several haplotypes (H5 for nDNA and H6-H7 for pDNA; Fig. 2). Therefore, our molecular phylogenies do not allow us to assess speciation events among the African rain forest species, suggesting they have diverged recently and/or are in the process of introgressing.

The observed nuclear/plastid incongruence could have resulted from incomplete lineage sorting or from hybridisation events. The latter could be linked to polyploid formation involving more than one species. Many alternative scenarios could be conceived to explain the origin of polyploidization in this genus (e.g. involving unreduced gametes, triploids, Petit et al., 1999; Burton and

Table 4
Characteristics and polymorphism of the four plastid and two nuclear DNA regions sequenced for *Afzelia* taxa.

Locus	Number of samples amplified	Alignment length	Number of Indels	Polymorphic sites	Parsimony-informative sites	Model of evolution
ITS2[a]	15	471	0	125 (26.5%)	73 (58.4%)	HKY + G
PEPC E7[a,b]	31	558	0	31 (5.5%)	14 (45.2%)	K80 (2nd) & JC (1st & 3rd codon positions)
psbA[a]	31	368	10	15 (4.1%)	13 (86.7%)	F81
ndhF[a]	31	486	0	2 (0.4%)	2 (100.0%)	F81
trnL[a]	31	432	9	5 (1.2%)	3 (60.0%)	F81
Plastome[b]	9	174,255	n	4370 (2.5%)	692 (15.8%)	TVM + G

[a] Phylogeny with MrBayes and Network.
[b] Molecular dating with BEAST; n: All missing data are replaced by N in plastome sequences.

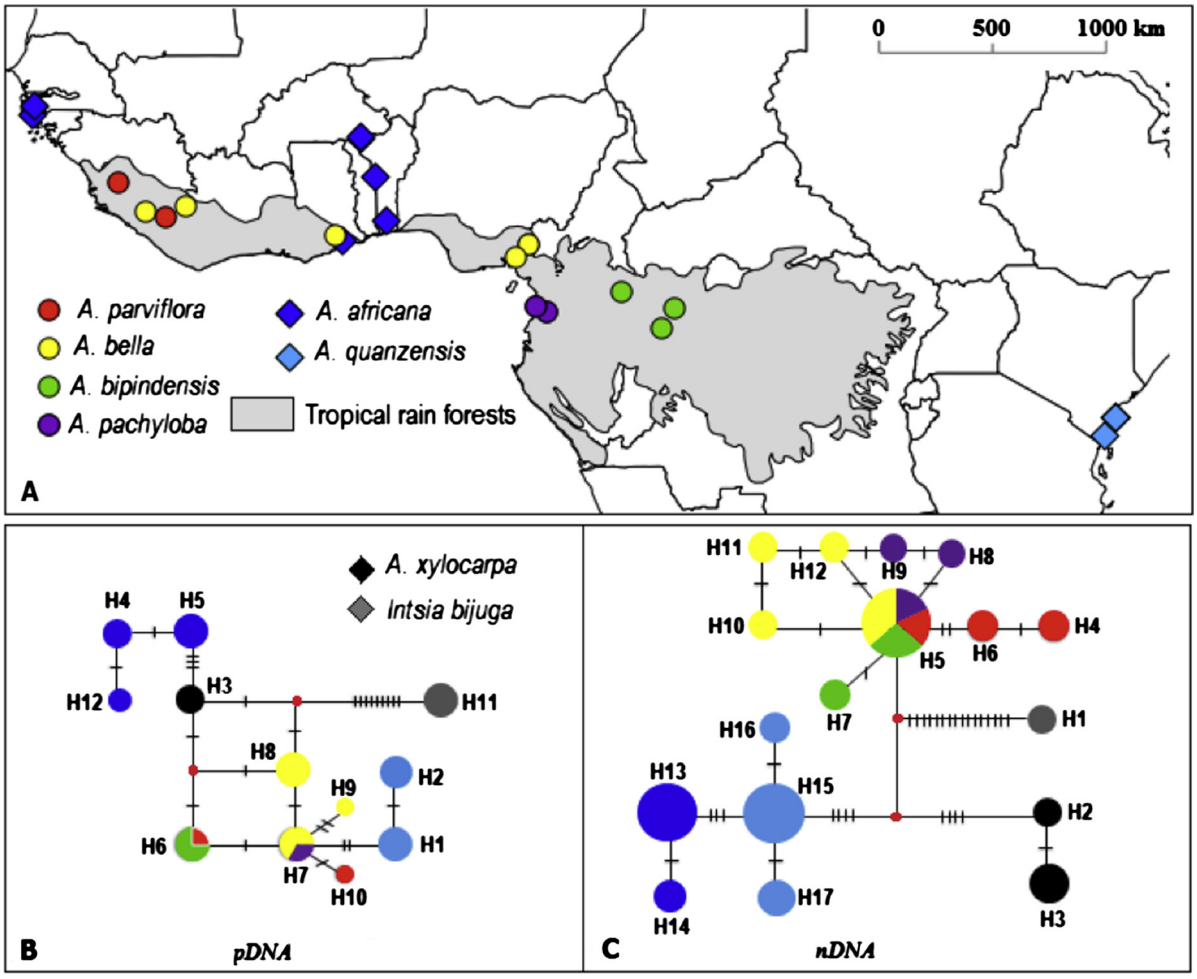


Fig. 2. Location of sequenced samples of African *Afzelia* species (A), and Median-joining haplotypes networks based on the plastid DNA concatenated regions *ndHF84F-ndHF713R*, *psbA-trnH* and *trnL*-intron (B) and on the nuclear *PEPC* E7 gene (C). Haplotypes in dark grey and black represent the outgroup taxon *Intsia bijuga* and the Asian *Afzelia xylocarpa*, respectively. Black dashes represent the number of mutations along each branch.

Husband, 2000) however, only a much more detailed analysis of the nuclear genome would bring additional insights. What can be confirmed, at least for the tetraploids, is that (i) they are closely related between themselves and did not derive from *A. africana*, (ii) an ancestor of *A. quanzensis* would have provided their plastid genome but not their nuclear genome, (iii) there is no evidence of a multiple origin of the tetraploids (although we cannot completely exclude it with current data).

4.2. Timing of diversification of the genus Afzelia and rain/dry forest transitions

According to the plastome and *PEPC* E7 phylogenetic trees, time-calibrated by 27.2 Ma old fossils attributed to *Afzelia* (Pan et al., 2010), the diversification of the genus leading to extant species seems to be concentrated in two phases: (i) the divergence between *A. africana*, the Asian *Afzelia* species, and the clade including tetraploid species in the early or mid-Miocene, from 12 to 15 Ma (plastomes) or 16–20 Ma (*PEPC* E7), (ii) the diversification of African tetraploid and wet forest species, initiated in the late-Miocene around 7.0 Ma (plastome, 95% HDP 2.8–15.1 Ma) or 9.4 Ma (*PEPC E7*, 95% HDP 3.5–18.8 Ma). In fact, our gene trees do not allow us to assess speciation times between tetraploid species because they are paraphyletic and share many haplotypes. However, our gene trees are still indicative of the time frame of diversification of the tetraploids taken as a whole. It is interesting to note that a fossil fruit from Uganda attributed to the tetraploid *A. bipindensis* has been dated around 5–6.5 Ma (Dechamps et al., 1992), which is compatible with the MRCA found for the tetraploid species. This tempo of diversification of the genus *Afzelia* is consistent with evidence from several plant and animal lineages suggesting that Guineo-Congolian rain forest species originated after 10 Ma (Plana, 2004). One classical hypothesis to explain the diversification of tropical species is that past climate changes (e.g., the progressive global cooling since the Miocene; Zachos et al., 2001), drastically modified the distribution of ecosystems and could have favoured species diversification, as hypothesised in Leguminosae (Pfeil et al., 2005; Cannon et al., 2010). In *Erythrophleum*, another Leguminosae genus that includes African rain forest and dry forest species as well as Asian species (all diploid), Duminil et al. (2015) reported a diversification during the late-Miocene and Pliocene (3–8 Ma) using plastid and nuclear gene trees. However, these authors detected a more recent diversification (late-Pliocene and Pleistocene, 0.6 to 4 Ma, for the African species) when they reconstructed a species tree using several nuclear genes. This contradiction between species trees and gene trees could be explained by the slow rate of coalescence in organisms with a long generation time, such as trees, favouring paraphyly.

Afzelia is included in a clade of mostly Asian tropical tree species (Bruneau et al., 2008), so African *Afzelia* may originate from Asia. However, Asian *Afzelia* do not occupy the most basal position in the phylogenies, suggesting forth and back migrations between

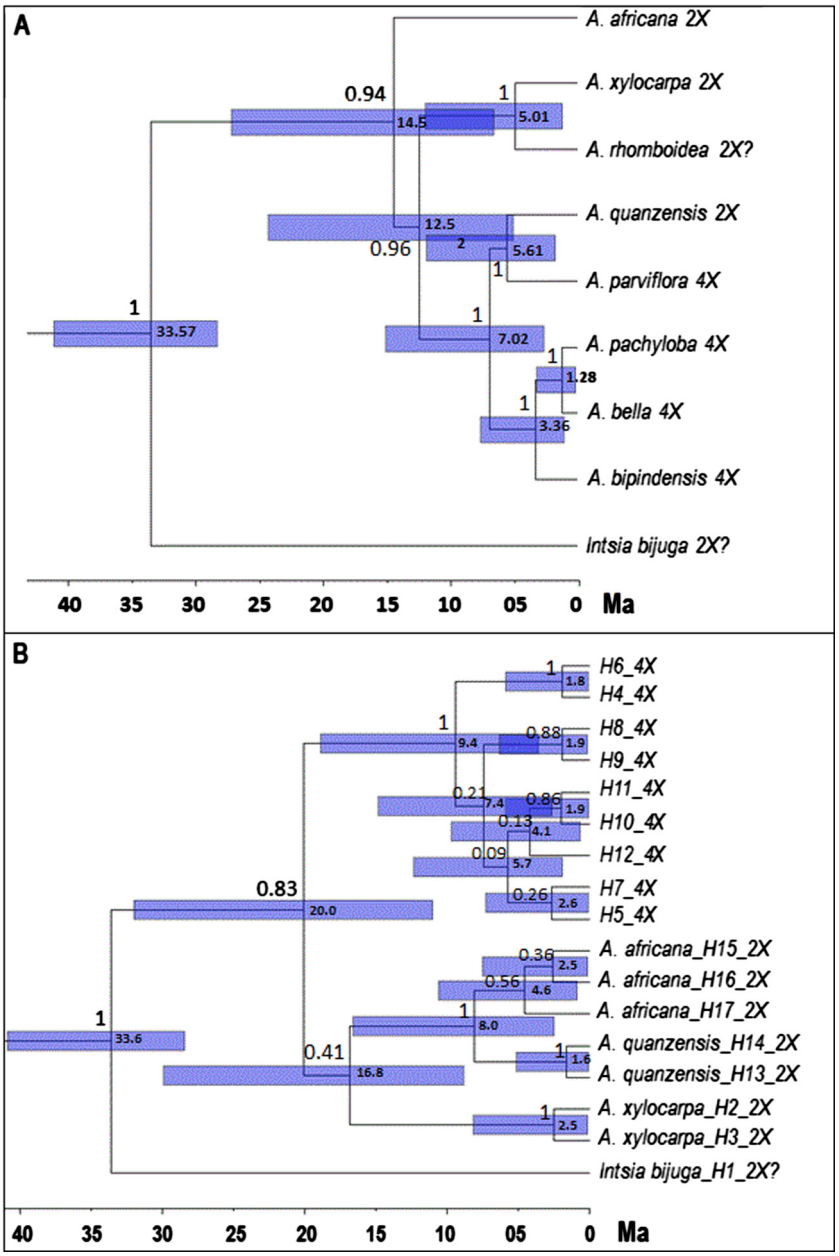


Fig. 3. Divergence time chronograms of the whole plastomes (A) and the nDNA PEPC E7 haplotypes (B, see Fig. 2C for corresponding haplotype numbers) sampled in *Afzelia* species (Bayesian maximum clade credibility trees). Numbers on nodes indicate the mean divergence time estimate (in Ma) and numbers on branches the branch support (posterior probability). Bars indicate the 95% highest posterior density intervals around node ages. The names of tetraploid species have been omitted from panel B because they share some of the *PEPC* E7 haplotypes. In fact, as the four tetraploid species (*4X*) share both pDNA and nDNA haplotypes (Fig. 2B and C), divergence times between tetraploid samples should not be interpreted as the corresponding divergence times between tetraploid species.

Asia and Africa, although data on more loci would be required to test this idea. The alternation of cold and warm periods could explain successive contraction and expansion of rain forests, with persistence in refuge areas (Maley, 1991, 1996; Dauby et al., 2014). Such phenomenon may have acted as a speciation pump for tetraploids but more detailed phylogeographical data are needed to confirm this hypothesis. The fragmentation induced by past Quaternary climate changes could have favoured the habitat shift between rain and dry forests species, combined with the phenomenon of polyploidization. However, if tetraploids have a unique origin as supported by our data, the correlation between ploidy and dry/rain forest habitat may be spurious and due to niche conservatism after an initial niche diversification event coinciding with the polyploidization. As diploid dry forest species are basal while tetraploid rain forest species form a derived clade according to nuclear data, the rain forest *Afzelia* species could originate from dry forest species. However, an alternative scenario with a rain forest dwelling ancestor cannot be excluded. Firstly, we can imagine a polyploidization scenario where the nuclear genome of tetraploids may derive from an extinct diploid species that could have already been adapted to rain forests. Secondly, Asian *Afzelia* and the sister genus *Intsia* are not dry forest trees (Asian *Afzelia* species occur in deciduous or inundated forests, *Intsia* species are found in coastal or mangrove forests). Thirdly, the *Afzelia* fossil of 27 Ma discovered in Ethiopia came from a riparian forest ecosystem (Pan et al., 2010).

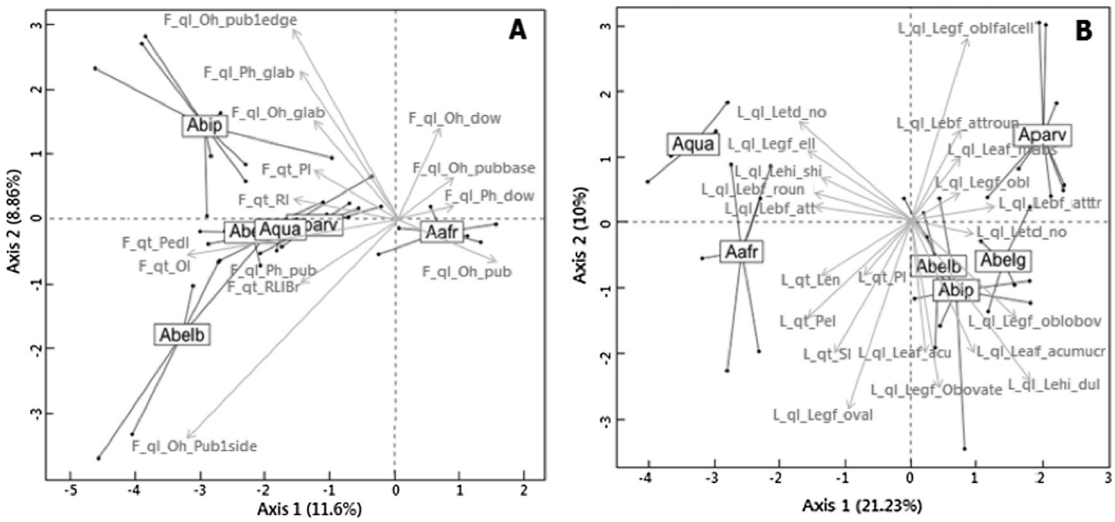


Fig. 4. Morphological variability of 38 specimens of African *Afzelia* for seven floral characters (A) and 15 vegetative characters (B), using the two first principal coordinates (PCoA, Hill-Smith analysis). Abip: *A. bipindensis;* Abelb: *A. bella var bella*; Abelg: *A. bella var gracilior*; Aparv: *A. parviflora*; Aafr: *A. Africana;* Aqua: *A. quanzensis*. See Table 3 for codes of morphological characters.

Table 5
Mean ± standard deviation [range] of 13 quantitative morphometric variables measured in six *Afzelia* taxa. Significant differences of means (10 normally distributed variables) or medians (three not normally distributed variables, grey shading) among taxa tested using Tukey-Kramer HSD and Kruskal-Wallis tests, respectively. Taxa that do not differ significantly share a common subscript letter. See Table 3 for abbreviations.

	$F_{4.33}$	Pvalue	*A. Africana* (6)	*A. quanzensis* (5)	*A. bella var bella* (5)	*A. bella var gracilior* (4)	*A. bipindensis* (9)	*A. parviflora* (9)
L_qt_Ll	16.6	<0.0001***	$29.17^{a} \pm 4.86$ [22.5–35]	$24.94^{ab} \pm 1.60$ [22.5–27]	$23.00^{b} \pm 2.32$ [20.5–26]	$19.50^{bc} \pm 3.49$ [15.5–24]	$23.30^{b} \pm 2.19$ [16.5–26.5]	$15.67^{c} \pm 2.87$ [12.5–21]
L_qt_Sl	17.1	<0.0001***	$25.20^{a} \pm 4.50$ [18.7–30]	$20.98^{ab} \pm 1.04$ [20–22.5]	$19.30^{b} \pm 1.90$ [17.5–22]	$16.25^{bc} \pm 3.12$ [13–20.5]	$20.35^{b} \pm 1.73$ [18–23.5]	$13.17^{c} \pm 2.56$ [10–18]
L_qt_Pl	5.0	0.0017*	$3.97^{a} \pm 0.55$ [3.5–5]	$3.96^{a} \pm 0.94$ [2.5–5]	$3.60^{ab} \pm 0.89$ [3–5]	$3.76^{ab} \pm 0.63$ [2.5–4]	$2.94^{ab} \pm 0.68$ [2–4]	$2.50^{b} \pm 0.54$ [1.5–2.8]
L_qt_NSV	7.4	<0.0001***	$8.50^{a} \pm 1.38$ [7–10]	$7.40^{a} \pm 0.89$ [6–8]	$10.80^{bc} \pm 1.30$ [9–12]	$9.50^{abc} \pm 0.58$ [9–10]	$10.78^{b} \pm 0.97$ [10–13]	$9.00^{ac} \pm 1.50$ [7–11]
L_qt_Pel	9.3	<0.0001***	$7.50^{a} \pm 1.18$ [6.5–9]	$6.36^{ab} \pm 1.96$ [4.5–8.5]	$5.60^{ab} \pm 1.02$ [4–6.5]	$4.08^{bc} \pm 0.65$ [3.5–5]	$5.06^{ab} \pm 1.47$ [3–7.5]	$3.46^{c} \pm 0.74$ [2.5–4.5]
L_qt_RlLl	9.9	<0.0001***	$1.74^{ab} \pm 0.21$ [1.5–2.04]	$1.46^{b} \pm 0.15$ [1.4–1.7]	$1.76^{ab} \pm 0.23$ [1.4–2]	$2.40^{c} \pm 0.43$ [1.8–2.8]	$1.87^{a} \pm 0.16$ [1.6–2.05]	$1.98^{a} \pm 0.15$ [1.8–2.04]
L_qt_RSLl	4.5	0.0034*	$2.00^{a} \pm 0.25$ [1.6–2.4]	$1.96^{a} \pm 0.19$ [1.7–2.1]	$2.38^{ab} \pm 0.25$ [2.1–2.7]	$2.50^{b} \pm 0.26$ [2.2–2.8]	$2.40^{b} \pm 0.29$ [2.02–2.8]	$2.23^{ab} \pm 0.20$ [1.8–2.4]
F_qt_Pl	21.5	<0.0001***	$16.58^{a} \pm 2.37$ [14–19.5]	$33.50^{c} \pm 2.83$ [31–38]	$54.00^{b} \pm 5.40$ [48.5–61]	$44.63^{bc} \pm 5.00$ [41–52]	$40.80^{c} \pm 7.00$ [33–52]	$40.06^{c} \pm 9.12$ [28–59]
F_qt_Pedl	2.3	0.0663^{ns}	$8.00^{a} \pm 1.30$ [6.5–10]	$8.40^{a} \pm 2.07$ [7–12]	$9.66^{a} \pm 1.33$ [8.8–12]	$8.65^{a} \pm 2.62$ [5–11]	$10.22^{a} \pm 1.28$ [8.5–12]	$8.11^{a} \pm 1.50$ [6.5–11.5]
F_qt_Rl	9.7	<0.0001***	$8.65^{a} \pm 2.25$ [5–11.5]	$17.46^{bc} \pm 4.40$ [11–21.5]	$19.16^{bc} \pm 2.05$ [15.5–20.3]	$18.00^{bc} \pm 2.34$ [16.5–21.5]	$20.22^{b} \pm 5.21$ [12–28.5]	$14.67^{c} \pm 1.70$ [12.5–17.5]
	χ^2_5							
L_qt_Len	17.0	0.0045*	$4.50^{ab} \pm 0.55$ [4–5]	$6.00^{a} \pm 0.55$ [5–6]	$4.00^{ab} \pm 0.84$ [3–5]	$5.50^{ab} \pm 0.96$ [4–6]	$5.00^{ab} \pm 0.83$ [4–6]	$4.00^{b} \pm 0.60$ [3–5]
F_qt_RLlBr	9.2	0.0999^{ns}	$1.30^{a} \pm 0.18$ [1.2–1.7]	$1.60^{a} \pm 0.24$ [1.1–1.7]	$1.80^{a} \pm 0.40$ [1.3–2.1]	$1.40^{a} \pm 0.20$ [1.1–1.6]	$1.40^{a} \pm 0.18$ [1.05–1.6]	$1.30^{a} \pm 0.16$ [1.1–1.3]
F_qt_Ol	15.9	0.0072*	$6.35^{a} \pm 1.05$ [5.5–8.5]	$14.50^{b} \pm 4.42$ [8–18.5]	$9.50^{ab} \pm 1.30$ [8.5–11.5]	$10.00^{ab} \pm 7.30$ [6.5–23]	$12.00^{b} \pm 2.34$ [8–12.5]	$8.80^{ab} \pm 2.07$ [5.5–12]

*** $P \leq 0.0001$; ** $0.0001 < P \leq 0.001$; *$0.001 < P \leq 0.05$; ns $P \geq 0.05$.

4.3. Genetic and morphological species delimitation in Afzelia

From the patterns obtained from both plastid and nuclear markers, we find that each diploid species is monophyletic with high posterior probabilities while tetraploid species form a monophyletic group whose internal resolution is so shallow that not a single clade can be recognized and several tetraploid species share nDNA or pDNA haplotypes (Figs. 2 and 3). This low potential of pDNA and nDNA to discriminate tetraploid species could be due to taxonomic problems in species delimitation and/or to the retention of ancestral polymorphism. It is worth noting that the time needed to reach reciprocal monophyly can be very long in long-living organisms like canopy trees, and this effect is reinforced in polyploids. Indeed, coalescence theory shows once a diploid population splits in two reproductively isolated populations (speciation event) of effective size *Ne*, at least 5 *Ne* generations are needed to reach reciprocal monophyly at most nuclear genes (Hudson and Coyne, 2002). In the case of an outbreeding autotetraploid (tetrasomic inheritance), *c.* 10 *Ne* generations are needed. Thus, assuming a *Ne* of at least 5000 and a mean generation time of ca. 200 years, it is not unlikely that million(s) of years may be needed after a speciation event before reaching reciprocal monophyly in

our tetraploid species. Therefore, species can be paraphyletic, similar to what Dainou et al. (2014) as well as Pennington and Lavin (2016) found in their species delimitation studies.

Our morphological analyses (with vegetative traits) supported that *Afzelia* species can be ordinated into two major groups, congruent with those revealed by our nDNA molecular phylogeny. Despite the morphological differences between dry and rain forest species, they have similarities on floral and seeds traits (data on colour of petals and seeds characteristics not available in herbarium), related potentially to similar pollination mechanisms. Overall the tetraploid species do not appear well differentiated morphologically (with the exception of *A. pachyloba*, not included in our analysis due to a lack of fertile material, but characterized by small leaflets).

5. Conclusion

This work is the first, to our knowledge, to assess whether polyploidization could be involved in habitat shift in tropical trees. Our genetic and morphological data on African *Afzelia* species indicate that while diploid species are well delimited, this is not the case for most of the tetraploid species. This pattern is coherent with our molecular dating suggesting that tetraploids diversified more recently than diploids. African *Afzelia* show a strong association between species diversification, polyploidy and habitat specialisation, suggesting that the differentiation between the savannah-dwelling diploid species and the forest-dwelling tetraploid species might be related to ecological gradients or past environmental changes. However, because polyploids form a single clade, this ploidy-habitat association lacks phylogenetic independence and no causal link can be inferred. Additional studies on tropical genera with varying ploidy levels are needed to test if polyploidization can facilitate a shift of habitat in the tropics, as was shown for example in arctic regions (Brochmann et al., 2004).

Data accessibility

DNA sequences have been deposited in GenBank under the following accession numbers: *psbA/trnH*: KX657797-KX657827; *ndHF84F/ndHF713R*: KX690241-KX690271; *trnL*: KX690210-KX690240; *PEPC* E7: KX690272-KX690288; *ITS2*: KX690289-KX690303; Plastomes: KX673213-KX673214.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.11.004.

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